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Methionine Adenosyltransferase:Adrenergic-cAMP Mechanism Regulates a Daily Rhythm in Pineal Expression*

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(*S*)-Adenosylmethionine (SAM) is a critical element of melatonin synthesis as the methyl donor in the last step of the pathway, the *O*-methylation of *N*-acetyl 5-hydroxytryptamine by hydroxyindole-*O*-methyltransferase. The activity of the enzyme that synthesizes SAM, methionine adenosyltransferase (MAT), increases 2.5-fold at night in the pineal gland. In this study, we found that pineal MAT2A mRNA and the protein it encodes, MAT II, also increase at night, suggesting that the increase in MAT activity is caused by an increase in MAT II gene products. The night levels of MAT2A mRNA in the pineal gland were severalfold higher than in other neural and non-neural tissues examined, consistent with the requirement for SAM in melatonin synthesis. Related studies indicate that the nocturnal increase in MAT2A mRNA is caused by activation of a well described neural pathway that mediates photoneural-circadian regulation of the pineal gland. MAT2A mRNA and MAT II protein were increased in organ culture by treatment with norepinephrine (NE), the sympathetic neurotransmitter that stimulates the pineal gland at night. NE is known to markedly elevate pineal cAMP, and here it was found that cAMP agonists elevate MAT2A mRNA levels by increasing MAT2A mRNA synthesis and that drugs that block cAMP activation of cAMP dependent protein kinase block effects of NE. Therefore, the NE-cAMP dependent increase in pineal MAT activity seems to reflect an increase in MAT II protein, which occurs in response to cAMP→protein kinase-dependent increased *MAT2A* expression. The existence of this MAT regulatory system underscores the importance that MAT plays in melatonin biogenesis. These studies also point to the possibility that SAM production in other tissues might be regulated through cAMP .

(*S*)-Adenosylmethionine (SAM),[1] the universal methyl donor, is synthesized by methionine adenosyltransferase (MAT; EC 2.5.1.6) (1–3). MAT exists in three forms, designated MAT I, MAT II, and MAT III. These three isoforms are the products of two genes (*MAT1A* and *MAT2A*) (4). MAT I and MAT III are expressed only in adult liver and are encoded by the *MAT1A* gene (5). MAT I is a tetramer and MAT III is a dimer of a single α1-subunit. In contrast to the restricted distribution of MAT I and III, MAT II is found in fetal hepatic tissue, adult extrahepatic tissues, and some transformed hepatocytes. Encoded by *MAT2A*, the α2 subunit plays the role of catalysis; the entire enzyme comprises only α2 or is constructed with regulatory β-subunits (6). The function of the β-subunit seems to be to down-regulate MAT II activity (7); however, the precise nature of the interaction between the α2- and β-subunits is still not clear.

MAT in the pineal gland is of special interest because synthesis of the hormonal product of this tissue, melatonin, requires SAM at the last step (*i.e.* the *O*-methylation of *N*-acetyl 5-hydroxytryptamine by hydroxyindole-*O*-methyltransferase) (8–10). Melatonin synthesis in the pineal increases at night by ~10-fold in response to stimulation by a neural pathway that includes the central oscillator in the hypothalamus, the suprachiasmatic nucleus (SCN) (11). Signals from the SCN are transmitted to the pineal gland by a pathway that passes through central and peripheral neural structures to a subset of cells in the superior cervical ganglia (SCG); the final link is made by projections from these cells that pervade the pineal perivascular space. Release of norepinephrine (NE) from these projections at night leads to an increase in melatonin production (11). Light acts on this system in mammals *via* the eyes to synchronize the SCN clock to environmental lighting cycles, which in turn synchronizes all circadian rhythms. In addition, light acts downstream of the clock to gate SCN stimulation of the pineal gland; as a result, prolonged light exposure or a pulse of light at night prevents NE release from SCG projections in the pineal perivascular space, thereby blocking adrenergic stimulation of pinealocytes (11).

Stimulation of melatonin production is known to reflect

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[1] The abbreviations used are: SAM, (*S*)-adenosylmethionine; MAT, methionine adenosyltransferase; SCN, suprachiasmatic nucleus; SCG, superior cervical ganglia; SCGX, superior cervical ganglionectomy; NE, norepinephrine; AANAT, arylalkylamine *N*-acetyltransferase; Rp-8-PIP-cAMP, 8-piperidinoadenosine-3′,5′-cyclic monophosporothioate, sodium salt, Rp-isomer; SCGX, removal of superior cervical ganglia; ZT, zeitgeber time; PBS, phosphate-buffered saline; CAPS, 3-cyclohexylamino-1-propanesulfonic acid; PE, phenylephrine; PKA, protein kinase A; CRE, cAMP response element; RPL32, ribosomal protein L32.

changes in several elements of melatonin synthesis; the most dramatic are large night/day differences in the activity of the next-to-last enzyme in melatonin synthesis, arylalkylamine *N*-acetyltransferase (AANAT) (12). These control large changes in melatonin synthesis by controlling the production of *N*-acetyl 5-hydroxytryptamine. During the day, low levels of AANAT probably limit melatonin synthesis. At night, however, when the activity of this enzyme is high, maximal levels of melatonin synthesis probably reflect other elements in melatonin synthesis, including SAM production.

Studies of pineal SAM and MAT indicate that SAM levels decrease at night, apparently reflecting increased turnover and net consumption; in addition, MAT activity increases (13, 14), which may reflect a coordinated increase in SAM production in response to an increased requirement for melatonin synthesis. Our interest in MAT was stimulated by results of cDNA microarray analysis studies indicating that the expression of MAT2A mRNA increases at night in the pineal gland.[2] In this study, we focused on the factors controlling pineal MAT activity by measuring MAT activity, MAT II protein, and MAT2A mRNA. These results are of interest because they provide details of the regulation of pineal MAT activity and, by inference, SAM production; they are of broader interest because they provide a clue to understanding how SAM synthesis is regulated in other tissues and how it might be manipulated pharmacologically.

EXPERIMENTAL PROCEDURES

Materials

L-[*methyl*-^{3}H]Methionine and [^{32}P]αdCTP were obtained from Amersham Biosciences. L-(−)-Norepinephrine, *R*-(−)-phenylephrine, 8-bromo-cAMP, dibutyryl cAMP, (−)-isoproterenol, forskolin, actinomycin D, puromycin, $MgCl_2$, KCl, 2-mercaptoethanol, ATP, KT5720, Rp-8-PIP-cAMP, and methionine were purchased from Sigma (St. Louis, MO).

Animals and Tissue Preparations

Rats (male, Sprague-Dawley, 150–200 g) were obtained from Taconic Farms Inc. (Germantown, NY) (Figs. 1–3, 6–9); Panum Institute (Copenhagen, Denmark (Fig. 4); and Zivic-Miller Laboratories (Allison Park, PA) (Fig. 5). Surgical decentralization and removal of superior cervical ganglia (SCGX) were performed by Zivic-Miller Laboratories. Animals were housed for 2 weeks in lighting cycles of light/dark 14:10 (or 12:12 in the case of *in situ* hybridization studies). Animals were sacrificed by CO_2 asphyxiation and decapitated. For biochemical studies, glands were immediately placed on solid CO_2 and stored at −80 °C until use. "Day" pineal glands and tissues were removed at ~ZT7 or at the time indicated in the figures or text; "night" pineal glands and tissues were obtained at ~ZT19 or at the time indicated in the figures or text. Animals were killed in the "dark" using dim red light. For cytochemical studies, pineal gland or intact brain (with the pineal gland) were removed and frozen before sectioning. Animal use and care protocols were in accordance with NIH guidelines.

Organ Culture

Glands were incubated on a 6-mm diameter nylon mesh disk (37 °C; 95% O_2-5% CO_2) in 200 μl of Biggers, Gwatkin, Judah medium with Fitton-Jackson modification in a 24-well tissue culture plate (two glands per well; Ref .15). The glands were transferred to fresh media after 24, 36, and 48 h. At 48 h, the glands were transferred into a top-loading tabletop incubator, which allows direct access to wells through ports with minimal disturbance to temperature or gas phase. The glands were incubated under control conditions for 30 min and then transferred into medium containing drugs of interest. At the end of treatment, glands were placed in microtubes on solid CO_2 and stored at −80 °C until use. In experiments for which mRNA levels, protein levels, and enzyme activities are reported, each analysis was performed on separate sets of pineal glands treated in culture on the same day.

MAT2A mRNA

Northern Blot Analysis—Total RNA was extracted using the guanidine-HCl/phenol procedure (TRIzol reagent; Invitrogen). RNA was separated on a 1.5% agarose/0.7 M formaldehyde gel, transferred to a charged nylon membrane (Nytran; Schleicher and Schuell) by passive capillary transfer, and cross-linked to the membrane using ultraviolet light. The MAT hybridization probes used were based on the 3′ untranslated region of *MAT1A* (nucleotides 1283–1837; GenBank™ accession number X15734) or the 3′ untranslated region of *MAT2A* (nucleotides 2827–3348, GenBank™ accession number BC062394); the *AANAT* probe has been described previously (12). Probes were ^{32}P-labeled by random priming using a DNA Labeling kit (Amersham Biosciences). Blots were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (12), 18 S rRNA (nucleotides 484–1080; GenBank™ accession number M11188) or ribosomal protein L32 (RPL32) (nucleotides 1–408; GenBank™ accession number NM_013226) (16, 17) to monitor the quality of the RNA and equal loading. Blots were hybridized at 68 °C for 1.5 h in QuikHyb buffer (Stratagene, La Jolla, CA). The final wash was in 0.1× SSC/0.1% SDS at 60 °C for 20 min. Blots were visualized and quantitated using a PhosphorImager (Amersham Biosciences); values were normalized against the signals generated by 18 S rRNA, glyceraldehyde-3-phosphate dehydrogenase, or RPL32.

In Situ Hybridization of Brain Sections—15-μm sagittal cryostat sections of rat brains were cut and thaw mounted on Superfrost Plus glass slides. The slides were kept at −80 °C until processed. The sections were hybridized, as described previously (18), with an ^{35}S-labeled 32-mer oligonucleotide probe based on rat *MAT2A* sequence (5′-GCGTAACCAAGGCAATGTGCCATTGCGGCGTA; antisense strand, nucleotides 610–641; GenBank™ accession number BC062394). The probe was diluted in sterile diethyl pyrocarbonate-water to a concentration of 5.0 pmol/μl. Probe in 5 μl was then labeled with ^{35}S-ATP and terminal transferase (Roche) to a specific activity of 1×10^{18} dpm/mol.

Frozen tissue sections were then thawed, fixed for 5 min in 4% paraformaldehyde in PBS, washed twice for 1 min each in PBS, and acetylated (0.25% acetic anhydride in 0.9% NaCl containing 0.1 M triethanolamine for 10 min). The sections were then dehydrated in a graded series of ethanols and delipidated in 100% chloroform (5 min). They were partially rehydrated in 100 and 95% ethanol (1 min each) and allowed to dry.

For hybridization of the cryostat sections, labeled probe was diluted in the hybridization buffer (10 μl of labeled probe/ml hybridization buffer) consisting of 50% (v/v) formamide, 4× SSC (150 mM NaCl and 15 mM sodium citrate, pH 7.0), 1× Denhardt's solution (0.02% bovine serum albumin, 0,02% polyvinylpyrrolidone, 0.02% Ficoll) 10% (w/v) dextran sulfate, 10 mM dithiothreitol, 0.5 mg/ml salmon sperm DNA, and 0.5 mg/ml yeast tRNA. An aliquot of 200 μl of the labeled probe was pipetted onto each section. The sections were then covered with Parafilm and incubated in a humid chamber overnight at 42 °C. After hybridization, the slides were washed in 1× SSC four times for 15 min each at 55 °C, twice for 30 min each at room temperature, and rinsed twice in distilled water. The sections were dried and either exposed to an x-ray film for 1–2 weeks or dipped into an Amersham LM-1 emulsion and exposed for 2–4 weeks at 4 °C. The pineal hybridization signals in autoradiographs of sagittal sections were quantified using "Image 1.42" (Wayne Rasband, National Institutes of Health). Optical density was converted to disintegrations per minute per milligram of tissue using simultaneously exposed ^{14}C-labeled standards calibrated by comparison with ^{35}S-labeled brain-paste standards. Results are based on the analysis of pineal glands from four animals killed at night and four during the day. Four sections from each pineal gland were used to determine the strength of the signal; signals from different animals were normalized using a standard processed with the tissue.

In Situ Hybridization of Pineal Sections—The analysis of pineal sections was performed as described previously (19) using expressed sequence tags corresponding to *MAT2A* (GenBank™ accession number AI849149) and AANAT (GenBank™ accession number AW047378) as templates for generating cRNA probes, which were labeled with digoxigenin.

Analysis of MAT Protein

SDS-PAGE Electrophoresis—To prepare samples, two glands were homogenized by brief sonication (three 1-s pulses) using a Biosonik sonicator (Bronwill Scientific, Rochester, NY) in 50 μl of 0.1 M sodium phosphate buffer, pH 6.8, 4 °C; the homogenate was centrifuged

[2] MAT2A mRNA in the rat pineal gland was 5–6-fold higher at night relative to day in spotted cDNA array (n = 3) analysis (S. Blackshaw, S. L. Coon, J.-S. Kim, C. L. Cepko, and D. C. Klein, unpublished data). This was subsequently confirmed using an oligonucleotide array (Affymetrix; S. L. Coon, D. Carter, R. Baler, and D. C. Klein, unpublished data).

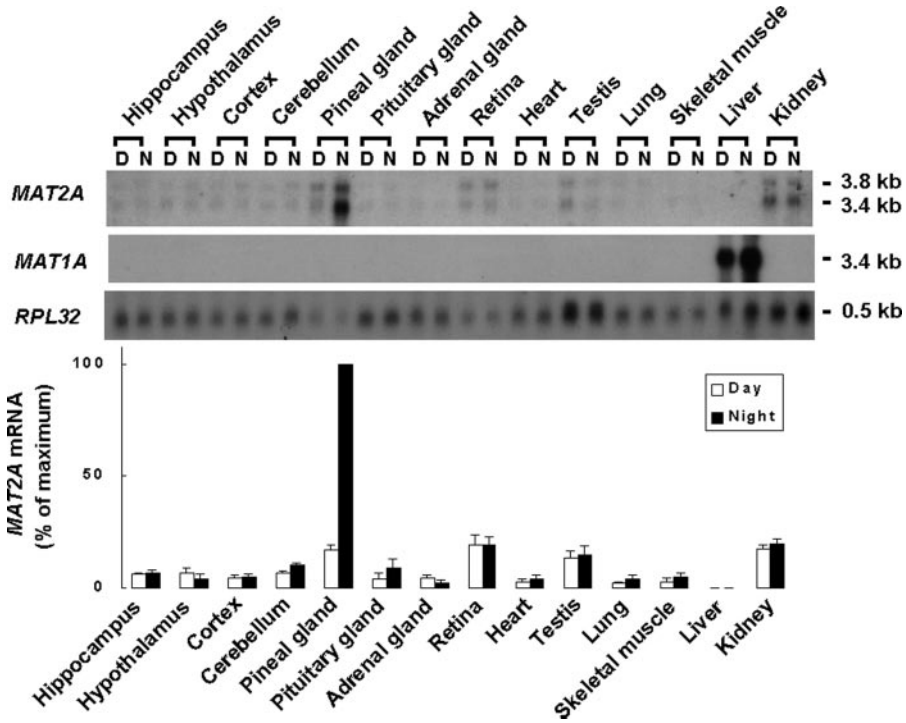

FIG. 1. **Rat MAT2A and MAT1A mRNA expression in select tissues at night and during the day.** Rats were housed in a controlled lighting environment (light/dark 14:10). Total RNA was obtained from day tissues removed at ZT 7 and night tissues removed at ZT 19 under dim red light. RNA preparation and Northern blot analysis were performed as described under "Experimental Procedures." The blot was hybridized with a rat *MAT1A* or *MAT2A* probe. To normalize RNA loading, blots were probed for *RPL32*. *D*, day; *N*, night. mRNA levels are expressed as a percentage of the maximum signal obtained in the pineal night samples. Results are mean ± S.D. of three replicates and reflect the combined values for both bands. For additional details, see "Experimental Procedures."

(13,000 × *g*, 15 min, 4 °C), and the supernatant was used for analytical procedures. Protein was measured by a dye binding method using a commercial reagent (Bio-Rad protein assay) with bovine serum albumin as the standard. Samples containing 60 μg of protein were resolved on pre-formed 10% Tris/glycine (1 mm) gels using the manufacturer's protocol (Novex, San Diego, CA). Rainbow standards (Amersham Biosciences) were used to determine the molecular mass of the proteins.

Electroblots—The proteins were electroblotted onto an Immobilon-P (0.45 μM) transfer membrane (Millipore, Bedford, MA) in a semi-dry blotting system (Investigator Graphite Electroblotter System; Genomic Solutions, Chelmsford, MA) according to the manufacturer's protocol. The proteins were equilibrated (5 min) and transferred using 10 mM CAPS buffer, pH 11, containing 20% methanol and 0.01% SDS. The conditions for electrical transfer were 400 μA/cm^2 (20 min), 600 μA/cm^2 (20 min), 800 μA/cm^2 (20 min), followed by 1200 μA/cm^2 (45min).

Immunodetection—Membranes were air-dried and then blocked for 2 h in PBS, pH 7.4, containing 10% non-fat dry milk (Bio-Rad), 0.2% Tween 20 (Bio-Rad), and 0.05% thimerosal (Sigma). An anti-MAT (6765) polyclonal was raised in New Zealand White rabbits against conjugated mouse MATII$_{95–119}$ (GenBank™ accession number A47151).

Antiserum was diluted (1:10,000) in PBS containing 1 mg/ml bovine serum albumin fraction V and 0.05% thimerosal. The membranes were incubated (18 h, room temperature) with antiserum and washed (twice for 5 min each in PBS containing 0.05% Tween 20 and then twice for 5 min each in PBS). The washed membranes were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (9 ng/ml, 1 h, room temperature; Kirkegaard and Perry Laboratories, Gaithersburg, MD) prepared in PBS containing 0.05% Tween 20, 0.05% thimerosal, and 2.5 μl/ml normal goat serum (Pierce). The membranes were then washed three times for 10 min each in PBS containing 0.05% Tween 20 followed by three 5-min washes in PBS. The secondary antibody was detected by enhanced chemiluminescence (Lumiglo; Kirkegaard and Perry). Loading was monitored by stripping the blot and reprobing with anti-actin serum (Sigma). The blots were exposed to BIO-MAX MR or X-Omat AR film (Eastman Kodak) and quantitated using ImageQuant 5.2 software (Amersham Biosciences).

MAT Enzyme Activity Measurement

MAT activity was measured by a modification of a method published previously (20). In brief, a pineal homogenate was prepared as described above, and a sample containing 40 μg of protein was incubated (45 min, 37 °C) in reaction mixture (100 μl) containing 0.1 M Tris-Cl, pH 8.1, 20 mM $MgCl_2$, 0.15 M KCl, 5 mM 2-mercaptoethanol, and 0.5 mM L-[*methyl*-^{3}H] methionine (0.5 μCi). The reaction was stopped by addition of 10 μl of 2 M $HClO_4$ containing 5 mM methionine. After centrifugation, 50 μl of the supernatant solution was spotted onto phosphocellulose filter paper (Whatman), washed twice for 10 min each in 5 mM potassium phosphate buffer, pH 7.5, and transferred to a counting vial containing 0.4 ml of 1.5 M NH_4OH. Radioactivity was measured in a scintillation counter.

In Vivo Isoproterenol Administration

Isoproterenol was dissolved in 0.85% NaCl to a final concentration of 10 mg/ml, and the appropriate volume (20 mg/kg rat) was injected subcutaneously at ZT4 or ZT7. Rats were killed at ZT7 or ZT11, and their pineal glands were removed and immediately placed on solid CO_2 and stored at −80 °C until use.

Statistical Analysis

Data are expressed as mean ± S.D. values for the number of determinations indicated. Statistical analyses were performed using Student's *t* test or Mann Whitney *U* test (21). In cases in which data from multiple blots or ISH images were subjected to statistical analysis, data from each source were normalized by setting the maximum values on each blot at 100% (*e.g.* night values); in these cases, S.D. values of the maximum values may not appear in the figures. A p value of <0.05 was considered significant.

RESULTS

MAT2A mRNA: Daily Rhythm and Tissue Expression—A daily rhythm in pineal MAT2A mRNA was clearly present in the pineal gland; night values were ~6-fold higher than day values (Figs. 1–5), confirming the results of microarray studies.[2] MAT2A mRNA levels during the day in the 14 tissues examined were similar. MAT2A mRNA was detected as two bands (~3.4 and 3.8 kb) (23, 24); MAT2A mRNA abundance did not exhibit a night/day difference in extra-pineal tissues. MAT1A mRNA was detected in the liver but in neither night nor day samples of the pineal gland or other tissues examined (Fig. 1), providing further confirmation of the liver-specific expression pattern of this gene (22, 23).

MAT2A mRNA levels increased significantly to maximum levels in the first 4–6 h of darkness at night (Fig. 2*A*), remained elevated for the duration of the night, and returned to day values within ~4 h of light. The maximum night/day difference in this study was ~6-fold, based on total signal. Analysis of the intensity of the two MAT2A mRNA bands revealed that although they both were prominent, there was a distinct

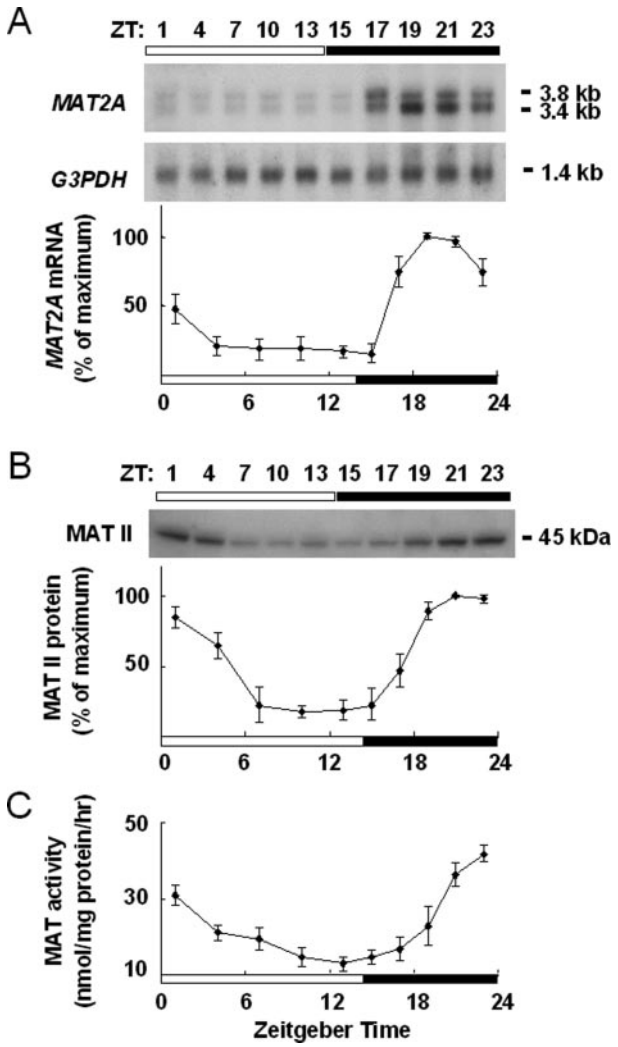

FIG. 2. **Daily rhythm in rat pineal MAT2A mRNA and of MAT II protein and activity.** Animals were housed in a controlled lighting environment for 2 weeks (light/dark 14:10). Pineal glands were prepared at the indicated time. *A*, MAT2A mRNA. Northern blot analysis was performed on total RNA obtained from each indicated ZT time as indicated under "Experimental Procedures." Each lane was loaded 10 μg of total RNA. To normalize RNA loading, the blot was reprobed for glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*). mRNA levels are expressed as a percentage of the maximum signal obtained in the ZT19 sample. Results are mean ± S.D. of three replicates. *B*, MAT II protein. Pineal extract was obtained at each indicated ZT time as indicated under "Experimental Procedures." Each lane was loaded with 60 μg of protein from a pineal extract. Immunodetection was performed with anti-MAT serum at a dilution of 1:10,000. Protein levels are expressed as a percentage of the maximum signal obtained in the ZT21 samples. Results are mean ± S.D. of three replicates. *C*, MAT activity. Pineal glands were obtained at the indicated ZT time. MAT enzyme activity was measure as described under "Experimental Procedures." Results are mean ± S.D. of three replicates. For further details, see "Experimental Procedures."

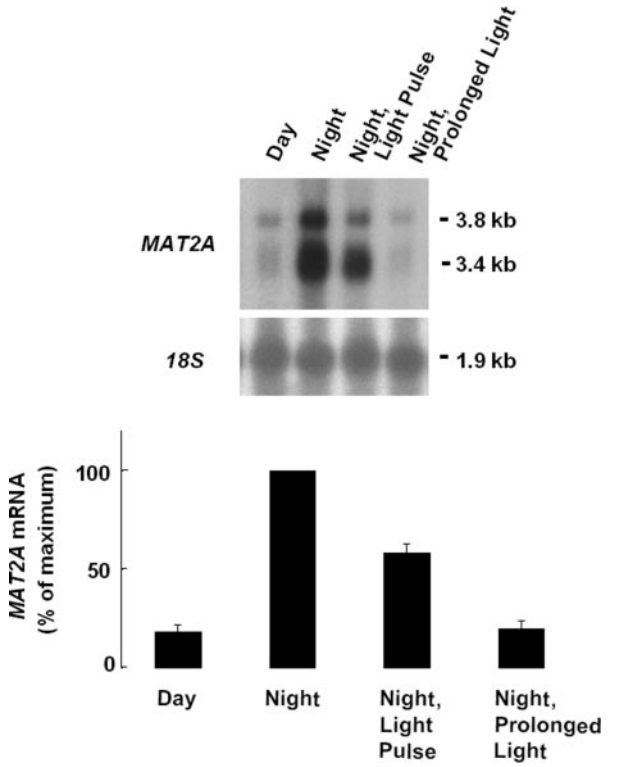

FIG. 3. **Effect of light exposure at night on MAT2A mRNA level in the rat pineal gland.** Rats were housed in a controlled lighting environment (light/dark 14:10). Pineal glands were obtained during the day (ZT 7), at night at ZT 19 in the dark, after a 30-min light pulse, or after 5 h of prolonged light. mRNA was extracted from three glands. RNA loading was normalized by reprobing the blot for 18 S rRNA. mRNA levels are expressed as a percentage of the maximum signal obtained at night sample. Results are mean ± S.D. of three replicates. For further details see "Experimental Procedures."

time-dependent difference in their relative abundance. The upper band was maximum at ~ZT 17 and the lower band at ~ZT 19; the latter was more intense.

The nocturnal increase in MAT2A mRNA was blocked by prolonged exposure to light at night (Fig. 3). In addition, a 30-min pulse of light at night caused MAT2A mRNA levels to decrease by ~50% ($p < 0.01$ compared with the night group), suggesting to us that there is a rapid turnover of MAT2A mRNA.

MAT2A mRNA Is Highly Expressed in Pinealocytes Relative to Brain—In situ hybridization for *MAT2A*, conducted on sagittal sections of the rat brain with the ^{35}S-labeled probe, revealed a strong pineal signal relative to the brain, with a clear night/day rhythm (Fig. 4*A*). The signal was also present in the stalk and deep pineal gland. The densitometric quantification of the signal in the pineal gland showed the night time signal (ZT 18) to be 6-fold higher than the day time signal (ZT 6; $p < 0.01$). Weak *in situ* hybridization signals were also observed in the medial habenular nucleus, granular layer of the cerebellum, layer II of the neocortex, and the pyramidal cells and granular cells of the hippocampal formation; night/day differences were not detected in extrapineal sites.

The hybridization signal in the pineal gland showed neither regional nor cellular variations. Hybridization done with the digoxigenin-labeled probe confirmed that MAT2A mRNA is strongly expressed by virtually all pinealocytes in the middle of the dark phase (ZT18), a pattern essentially identical to that of *AANAT* (Fig. 4*B*), indicating both were located in the melatonin-producing pinealocytes. Animals that had been treated with 6 h of constant light starting at ZT12 showed identical expression levels at ZT18 and ZT6 (data not shown), consistent with results presented in Fig. 3.

A 24-h Rhythm of MAT II Protein and MAT Enzyme Activity in the Rat Pineal Gland—MAT II protein was detectable in 13,000 × *g* supernatant pineal extracts as a single 45-kDa band (Fig. 2*B*); this was also seen in other tissues (data not shown). The apparent size is in general agreement with a published report (48 kDa; Ref. 24). The abundance of MAT II protein changed during the 24-hour period, with maximal amounts detectable 8 h after the light/dark transition, somewhat later than the peak in MAT2A mRNA. MAT protein decreased after the dark/light transition and achieved minimum values within 7 h. The maximum night/day difference in the abundance of MAT II protein was ~5-fold ($p < 0.01$). Moreover, the reported daily

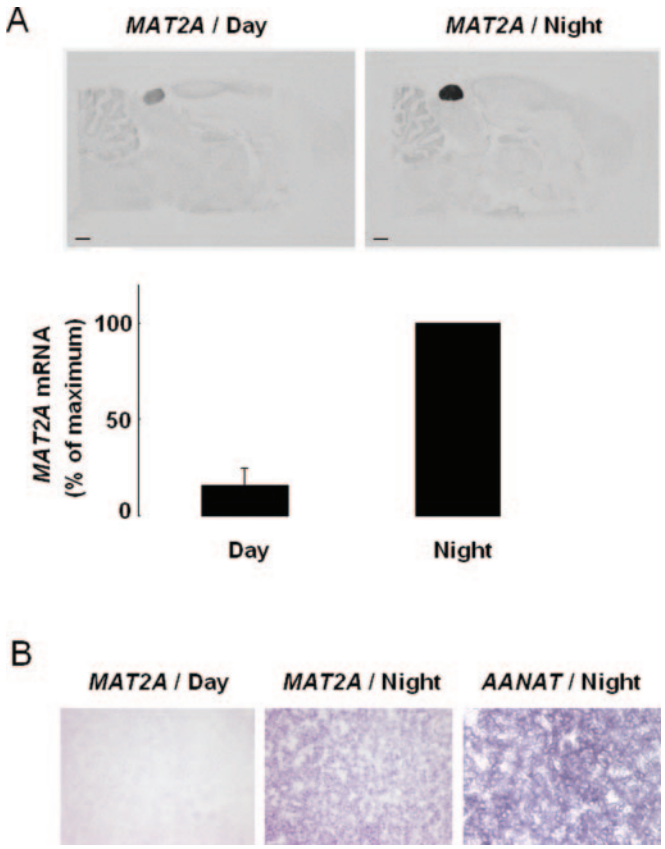

FIG. 4. ***In situ* hybridization of MAT2A mRNA.** Animals were housed in a controlled lighting environment for 2 weeks (light/dark 12:12). *A*, *in situ* hybridization signals of a median rat brain section that has been hybridized with an antisense probe for MAT2A mRNA. The *left section* is from an animal killed during the day (ZT 6), and the *right section* is from an animal killed during the night (ZT 18). *Scale bars*, 1 mm. Bar graph of the densitometric *in situ* signals of MAT2A mRNA of the pineal gland. MAT2A mRNA levels are expressed as a percentage of the maximum signal obtained at night (ZT18) sample. The nighttime value is ~6-fold higher than the daytime value. Results are mean ± S.D. from four rats with four sections from each rat. *B*, photomicrographs of sections of the rat pineal hybridized with digoxigenin-labeled probes (19). The signal was detected immunohistochemically. Virtually all pinealocytes express *MAT2A* at ZT18. Interstitial cells and endothelial cells were not positive for MAT2A mRNA. Three pineal glands were tested for each time point.

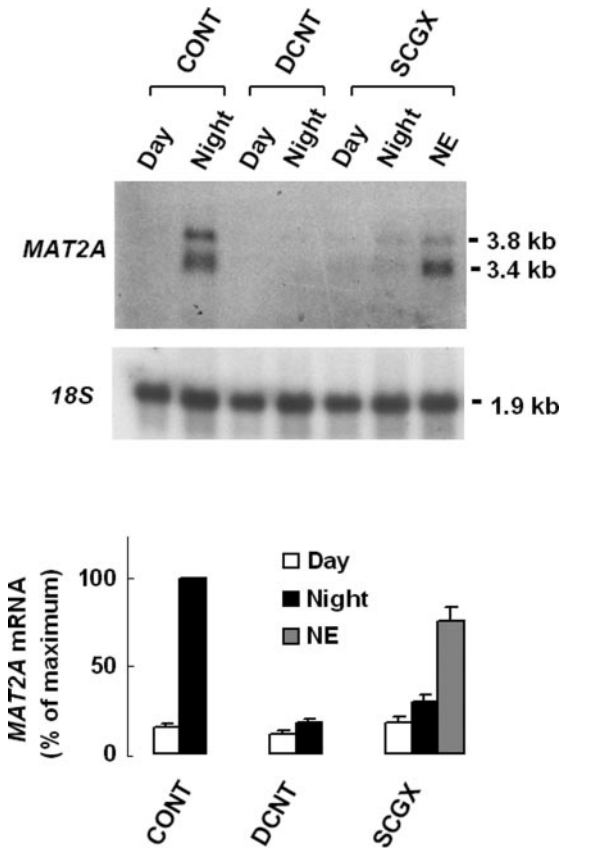

FIG. 5. **The nocturnal increase in MAT2A mRNA is driven by neural input from the SCG.** Pineal RNA was obtained from control (*CONT*), decentralized (*DCNT*), or SCGX rats killed at ZT7 (day) and ZT19 (night). In addition, one group of SCGX rats was injected with 1 mg/kg NE at ZT 16 and killed 3 h later with the other night experimental group. Each lane contains 3 μg of total RNA obtained from pools of two pineal glands. mRNA levels are expressed as a percentage of the maximum signal obtained in the control night sample. Results are mean ± S.D. of three replicates. For further details, see "Experimental Procedures."

rhythm in MAT activity (13) was confirmed in this study; the maximum night/day difference was ~4-fold (Fig. 2*C*; $p < 0.01$).

MAT2A mRNA Rhythm Is under Neural Control—To determine whether the daily rhythm in pineal MAT2A mRNA was neurally regulated, the effects of two lesions that block SCG-mediated neural stimulation were investigated: SCGX and decentralization of the SCG. The latter procedure leaves the SCG and SCG projections into the pineal gland intact. Both procedures blocked the nocturnal increase in MAT2A mRNA (Fig. 5; $p < 0.01$). These observations indicate that the increase in MAT2A mRNA is driven by SCG-mediated neural input to the pineal gland.

Adrenergic Regulation of MAT2A mRNA Transcription, MAT II Protein Synthesis, and MAT Activity—The adrenergic regulation of MAT2A mRNA and MAT activity was studied in the whole animal by examining the effect of treatment with adrenergic agonists. Injection of NE to SCGX animals elevated MAT2A mRNA >4-fold (Fig. 5; $p < 0.01$). In addition, injection of the β-adrenergic agonist isoproterenol (20 mg/kg) to intact animals also significantly elevated MAT2A mRNA and MAT activity (Fig. 6). These findings support the interpretation that the nocturnal increases in pineal MAT2A mRNA and MAT activity are caused by release of NE into the pineal extracellular space and that the action of NE involves β-adrenergic receptors. This does not, however, eliminate the possibility that other receptors may mediate similar effects.

These studies were extended using organ culture. Glands were incubated for 48 h, during which time sympathetic processes degenerate (25). Treatment of these glands with NE increased MAT2A mRNA, MAT protein, and MAT activity (Figs. 7 and 8; $p < 0.01$). This supports the conclusion that the physiological increases in these parameters reflect stimulation by NE (Fig. 7). Treatment with the α_1-adrenergic receptor agonist phenylephrine (PE) also increased MAT gene expression ($p < 0.01$), indicating that NE may be acting through both α_1- and β-adrenergic receptors.

cAMP Regulates MAT2A mRNA, MAT II Protein, and MAT Activity—NE is known to elevate cAMP and activate PKA in the pineal gland (11); therefore, it was of interest to investigate the effects of cAMP protagonists and PKA antagonists in organ culture. Treatment with forskolin (Fig. 7), which also elevates pineal cAMP, elevated MAT2A mRNA, MAT II protein, and MAT activity ($p < 0.01$). In agreement with these effects, it was also found that treatments with either of two cAMP analogs, 8-bromo-cAMP or dibutyryl cAMP, also significantly elevated MAT2A mRNA (Fig. 8*A*). PKA antagonists had opposite effects: KT5720 and Rp-8-PIP-cAMP inhibited the NE-induced MAT2A mRNA elevation (Fig. 8*B*). These findings support the conclusion that NE acts through a cAMP→PKA mechanism to alter

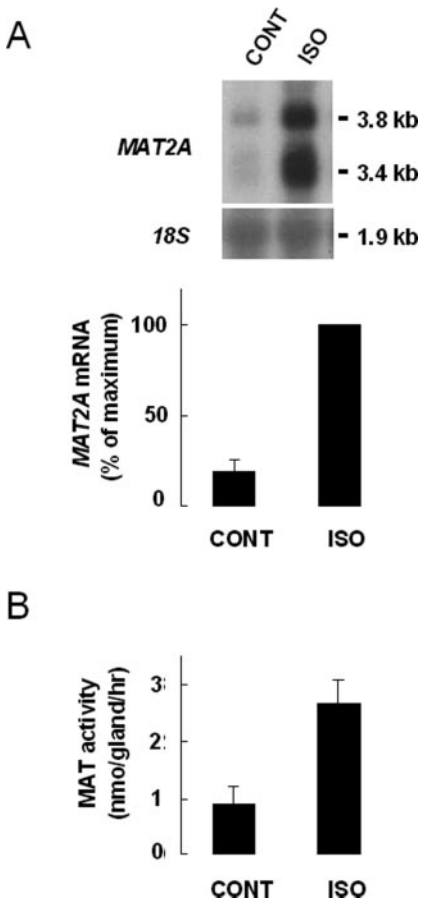

FIG. 6. **Isoproterenol (*ISO*) elevates MAT2A mRNA levels and activity *in vivo*.** *A*, rats were injected subcutaneously in the nape of the neck with a solution of isoproterenol (20 mg/kg in 0.4 ml of 0.85% NaCl) at ZT4. All animals were killed at ZT7, and their pineal glands were removed and stored on solid CO_2. Northern blots were probed with rat *MAT2A* probe. Each lane was loaded 5 μg of total RNA obtained from a pool of two pineal glands. To normalize for variations in RNA loading, the blot was reprobed for 18 S rRNA. mRNA levels are expressed as a percentage of the maximum signal obtained in the isoproterenol-injected sample. The results were confirmed in three independent experiments. Results are mean ± S.D. of three replicates. *B*, rats were injected subcutaneously in the nape of the neck with a suspension of isoproterenol (20 mg/kg in 0.4 ml of 0.85% NaCl) at ZT7. All animals were killed at ZT11, and their pineal glands were removed and frozen on solid CO_2 until protein extraction. Measurement of MAT II activity was performed as described under "Experimental Procedures." Results are mean ± S.D. of four replicates. For further details, see "Experimental Procedures."

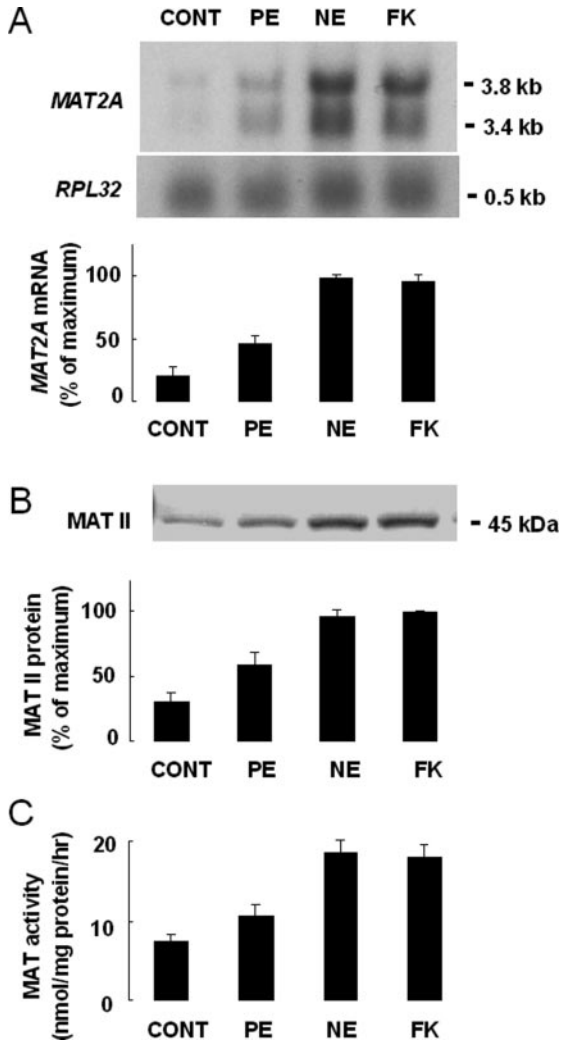

FIG. 7. ***In vitro* effects of adrenergic agonists and forskolin on MAT2A mRNA and of MAT II protein levels and activity.** Pineal glands were incubated for 48 h and then treated with 1 μM PE, 1 μM NE, or 10 μM forskolin (*FK*) for 6 h. *CONT*, control. At the end of treatment, glands were placed on solid CO_2 and stored at −80 °C until use. *A*, effects of PE, NE, and FK treatment on MAT2A mRNA expression. Each lane contained 7 μg of total RNA. Results are mean ± S.D. of three replicates. *B*, effects of PE, NE, and FK treatment on MAT II protein levels in cultured pineal gland. Each lane was loaded with 60 μg of protein from a pineal extract. Western blot analysis was done as indicated under "Experimental Procedures." Results are mean ± S.D. of three replicates. *C*, effects of PE, NE, and FK treatment on MAT II enzyme activity in cultured pineal glands. Results are mean ± S.D. of three replicates. For further details, see "Experimental Procedures."

MAT2A mRNA, which in turn leads to an increase in MATII protein and MAT activity.

NE-induced Elevation in MAT2A mRNA Requires Gene Transcription and Protein Synthesis—The issue of whether NE was acting to increase expression of *MAT2A* was examined by treating glands with NE in the presence or absence of an inhibitor of mRNA synthesis, actinomycin D. This treatment blocked the increase in MAT2A mRNA, indicating that the increase in MAT2A mRNA reflects increased transcription.

The role of new protein synthesis in the elevation of MAT2A mRNA was investigated using the protein synthesis inhibitor puromycin. This clearly reduced the intensity of the 3.4-kb MAT2A mRNA signal ($p < 0.01$ compared with the NE-treated group), indicating that the appearance of this band requires synthesis of a protein (Fig. 9*A*). Actinomycin D and puromycin were determined to be effective in this experiments because the NE-dependent increase in AANAT mRNA was enhanced by treatment with puromycin and blocked by actinomycin D (Fig. 9*A*), are previously reported (12). In addition, the NE-induced increase in MAT II protein was blocked by either actinomycin D or puromycin (Fig. 9*B*). This points to a requirement for *de novo* synthesis of both mRNA and protein for the observed effects of NE on MAT II protein.

DISCUSSION

The results presented indicate that MAT II is the only MAT isoform expressed in the pineal gland, as is the case in all tissues examined, other than the liver. *MAT2A* expression in the pineal gland, as judged by mRNA abundance, is notable for two reasons: 1) the marked nocturnal increase in mRNA levels and 2) the level of mRNA achieved at night is manyfold higher than expression in other tissues. Both of these characteristics (rhythmic expression and high levels of expression in the pineal gland) are not surprising in view of the central role of SAM in melatonin synthesis and the daily rhythm in synthesis.

These results also provide an understanding of the mecha-

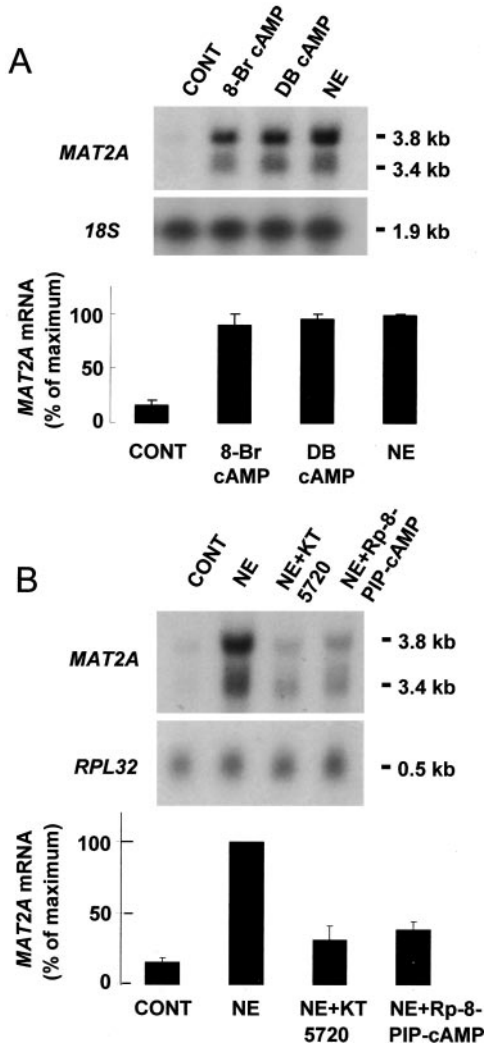

FIG. 8. ***In vitro* effects of cAMP protagonists and PKA antagonists on MAT2A mRNA.** Each lane contains 6 μg of total RNA obtained from a pool of four pineal glands. After hybridization with the *MAT2A* probe, the blot was stripped and reprobed with 18 S and RPL32 probes as a control. Results were confirmed in three independent experiments. mRNA levels are expressed as a percentage of the maximal signal obtained with 1 μM NE. Results are mean ± S.D. of three replicates. *A*, pineal glands were cultured for 6 h with 500 μM 8-bromo-cAMP (*8-Br cAMP*), 500 μM dibutyryl cAMP (*DB cAMP*), and 1 μM NE. *CONT*, control. *B*, pineal glands were cultured with 3 μM KT5720 or 500 μM Rp-8-PIP-cAMP for 1 h; NE (1 μM) was then added, and incubation was continued for 6 h. The indicated drugs were present during NE treatment.

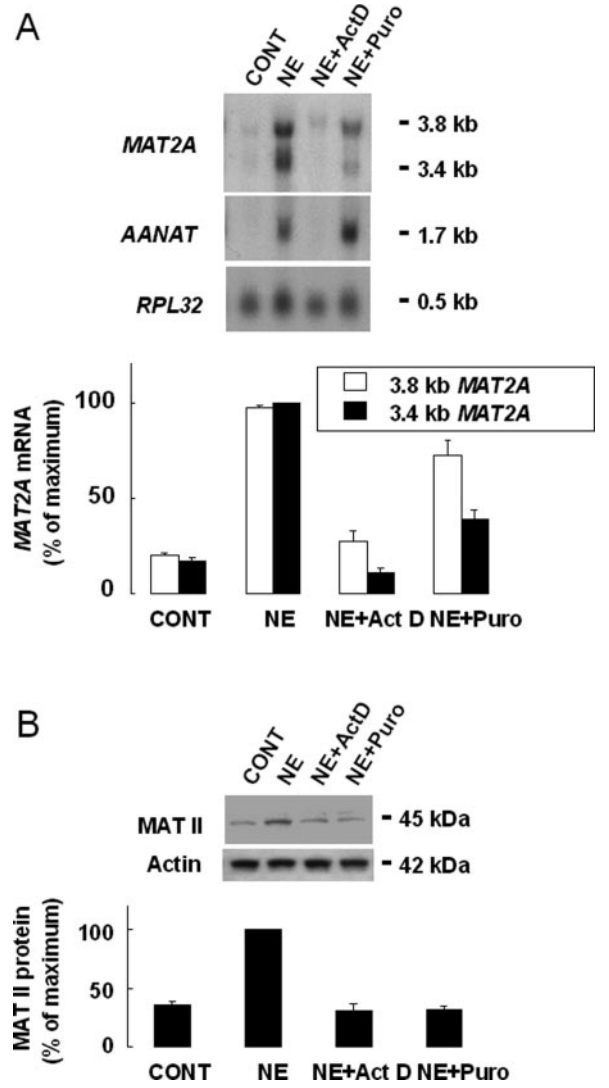

FIG. 9. **NE-induced elevation in MAT2A mRNA and protein requires gene transcription and protein synthesis.** Pineal glands were treated with 30 μg/ml actinomycin D (*Act D*) or 50 μg/ml puromycin (Puro) for 1 h and during the subsequent 6-h treatment with 1 μM NE. *A*, MAT2A and AANAT mRNA. Each lane was loaded 6 μg of total RNA obtained from a pool of four pineal glands. After hybridization with the *MAT2A* probe, the blot was stripped and reprobed with *AANAT* and *RPL32* probes as a control. Results were confirmed in three independent experiments. mRNA levels are expressed as a percentage of the maximal signal obtained with 1 μM NE. The values are mean ± S.D. of three replicates. *B*, MAT II protein. Each lane was loaded with 60 μg of protein from a pineal extract. Proteins were resolved by SDS-PAGE and subjected to immunoblotting with anti-MAT serum. To normalize, the blot was stripped and re-exposed to an anti-actin serum (Sigma). Protein levels are expressed as a percentage of the maximum signal obtained with 1 μM NE. Results are mean ± S.D. of three replicates. For further details, see "Experimental Procedures."

nism involved in regulating the nocturnal increase in *MAT2A* expression. This seems to be driven by neural stimulation *via* sympathetic innervation of the pineal gland, resulting in a release of NE; this in turn activates β-adrenergic-cAMP mechanisms that enhance *MAT2A* transcription. As discussed in the Introduction, rhythmic nocturnal neural stimulation of the pineal gland originates in the SCN, the site of the master circadian oscillator. Therefore, it is reasonable to suspect that the rhythm in *MAT2A* expression is truly circadian in nature.

This pattern of regulation applies to a number of genes in the rat pineal gland, including *arylalkylamine N-acetyltransferase*, *type II iodothyronine deiodinase*, *Id-1*, *inducible cAMP repressor*, and *Fos-related antigen-2*, in that photoneural and adrenergic-cAMP regulation has been demonstrated (12, 26–28). It is of interest to consider whether common transcriptional mechanisms are involved in the regulation of transcription of *MAT2A* and these genes. Preliminary evaluation of this suggests that different mechanisms are involved; specifically, the rat *AANAT* promoter contains cAMP response elements (CREs), which function to mediate cAMP→PKA→cAMP response element-binding protein control of expression in the pineal gland. In contrast, we have not found CREs in the 1000 bp located in the *MAT2A* promoter region (29) (MatInspector; Genomatix Software), suggesting that cAMP response element-binding protein may not play a direct role. On the other hand, it is also possible that CREs are located outside this region.

It is also possible that cAMP controls *MAT2A* through an indirect mechanism that might involve CRE-dependent generation of a transcription factor that interacts with another element in the *MAT2A* promoter. For example, cAMP stimulation of *MAT2A* transcription may be mediated by AP-1 sites in the

rat *MAT2A* promoter (29), in a manner similar to AP-1 mediation of cAMP stimulation of dopamine β-hydroxylase expression (30). In the pineal gland, cAMP treatment results in an elevation of c-Fos and Fos-related antigen-2, both of which bind to AP-1 sites. Either or both could mediate cAMP stimulation of *MAT2A* transcription (28, 31).

The observations that changes in MAT2A mRNA are translated with little delay into changes in MAT II protein *in vivo* and *in vitro* indicate that transcriptional regulatory mechanisms events seem to play a dominant role in controlling dynamic changes in the levels of protein encoded by the transcript, whereas translational and post-translational regulatory mechanisms may be of minor importance.

Our studies revealed an interesting difference in the effects of puromycin on the induction of the two bands of MAT2A mRNA (3.8 and 3.4 kb). Whereas this treatment did not significantly alter the increase in the abundance of the 3.8-kb band, it did dramatically reduce the abundance of the lower band. In view of the evidence that these bands represent splice variants of the same gene, the selective effect of puromycin on one band could be explained by the involvement of a rapidly turning over protein involved in splicing required for the 3.4-kb band to appear. The significance of the two bands of *MAT 2A* mRNA is not clear.

Some differences were observed between the dynamics of changes in MAT II protein and MAT activity. In particular, the night/day differences in enzyme activity were somewhat smaller, and MAT activity increased at a slower rate and reached a peak later than did MAT II protein. These differences may reflect undefined post-translational events that alter the activity of the protein, changes in the abundance of the β-subunit of MAT II, or a combination of these two factors.

Observations in the literature suggest that *MAT2A* is regulated differently in different tissues. For example, Zeng *et al.* (32) showed that treatment of Jurkat cells with phorbol 12-myristate 13-acetate resulted in a 2-fold increase in MAT2A mRNA levels and a 2-fold increase in luciferase activity driven by the transfected human *MAT2A* promoter construct, suggesting a role for protein kinase C. However, we found that treatment with phorbol 12-myristate 13-acetate alone had no effect on MAT2A mRNA levels in cultured pineal glands (data not shown). This does not eliminate the possibility that a protein kinase C mechanism is involved in some way in the control of *MAT2A* expression in the pineal gland, however. Protein kinase C is known to mediate the α-adrenergic potentiation of β-adrenergic stimulation of cAMP (11), raising the possibility that it acts by enhancing production of cAMP.

In addition, it has been found that *in vivo* injection of L-dopa to the mouse elevates expression and activity of MAT in the brain (24). Although dopamine is not known to play a transmitter role in pineal signal transduction, it is possible that administration of L-dopa could impact pineal function by increasing NE synthesis (L-dopa→dopamine→NE) and release at night. This could occur in Parkinson's disease patients receiving long-term high dose levels of L-dopa.

Our results indicate that the daily production of melatonin is accompanied by an increase in the expression of the *MAT2A* gene, which is translated into changes in MAT II protein and MAT activity. The evidence that MAT2A mRNA, MAT protein, and MAT activity change significantly on a physiological basis during a 24-hour period makes it clear that there is significant turnover in *MAT2A* gene products, reflecting rapid synthesis and degradation. As a result, it is possible also that SAM generation could be influenced by mechanisms that modulate the dynamic changes in any one of these parameters. This and the evidence that cAMP may control *MAT2A* and *AANAT* transcription through different mechanisms indicate that the regulation of the production of SAM and the expression of *AANAT* might be subject to differential regulation. Therefore, melatonin production might be altered through changes in SAM production without accompanying changes in AANAT activity.

The broader impact of these studies is the indication that the expression of *MAT2A* can be influenced by cAMP. This raises the possibility that SAM levels could be affected by treatments that modulate cAMP. This is of potential significance because of the broad importance of SAM in biology, especially in the nervous system, where it is required for the synthesis and degradation of neurotransmitters. It may also play a role in disease states (33–35). The development of potent and subtype-selective inhibitors of phosphodiesterase makes it increasingly feasible to use such inhibitors to selectively increase cAMP in target tissues to enhance SAM availability. In addition, monitoring *MAT2A* expression may be useful in evaluating the biological effects of such inhibitors.

The continued pursuit of the factors that are responsible for the high levels of expression of *MAT2A* in the pineal gland should lead to a better understanding of the regulation of this gene and eventually to strategies that pharmacologically modulate the levels of SAM in other tissues.

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